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In-situ substrate preferences of abundant bacterioplankton populations in a prealpine freshwater lake

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Abstract

The substrate partitioning of sympatric populations of freshwater bacterioplankton was studied via microautoradiography and fluorescence *in situ* hybridization. Fourteen radiolabelled tracers were used to assess microbial acquisition spectra of low-molecular-weight (LMW) organic compounds. The most abundant group, ac1 *Actinobacteria*, were highly active in leucine, thymidine, and glucose assimilation, whereas *Alphaproteobacteria* from the LD12 lineage (the freshwater sister clade of SAR11) only weakly incorporated these tracers, but exhibited a distinct preference for glutamine and glutamate. Different *Bacteroidetes* showed contrasting uptake patterns: *Flavobacteriales* did not incorporate significant amounts of any LMW compound, and *Cyclobacteriaceae* were clearly specialized on leucine, glucose, and arginine. *Betaproteobacteria* represented the most active and versatile bacterioplankton fraction and >90% of them could be assigned to 8 species- to genus-like populations with contrasting substrate specialization. *Limnohabitans* sp. were the most abundant and active *Betaproteobacteria,* incorporating almost all tracers. While three closely related betaproteobacterial populations substantially differed in their uptake spectra, two more distantly related lineages had very similar preferences, and one population did not incorporate any tracer. The dominant phototrophic microorganism, the filamentous cyanobacterium *Planktothrix rubescens,* assimilated several substrates, whereas other (pico)cyanobacteria had no heterotrophic activity. The variable extent of specialization by the studied bacterial taxa on subsets of LMW compounds contrasts theoretical considerations about non-selective microbial substrate assimilation at oligotrophic conditions. This physiological niche separation might be one explanation for the coexistence of freshwater bacterioplankton species in a seemingly uniform environment.

Introduction

The free-living bacterial assemblages in freshwater lakes are mainly composed of small *Actinobacteria* (esp. the acI cluster (Warnecke *et al.,* 2005, Allgaier and Grossart 2006)), of members of the LD12 cluster of *Alphaproteobacteria* (Salcher *et al.,* 2011b), of *Betaproteobacteria* (Glöckner *et al.,* 1999, Newton *et al.,* 2011), and diverse *Flavobacteria* and *Sphingobacteria* (Eiler and Bertilsson 2007, Newton *et al.,* 2011). Improved techniques such as filtration-acclimatization or dilution to extinction have allowed for the cultivation of numerically important freshwater *Betaproteobacteria* (*Limnohabitans* sp. ((Kasalicky *et al.,* 2010) and *Polynucleobacter* sp. (Hahn *et al.,* 2009, 2012a)), of *Flavobacteria* (e.g., *F. dankookense*, *F. aquatile* (Lee *et al.,* 2011), and of *Sphingobacteria* (e.g., *Algoriphagus aquatilis (Liu et al., 2009)*), while the most abundant freshwater bacteria (acI *Actinobacteria* and LD12 *Alphaproteobacteria*) still lack cultivated representatives. Therefore, cultivation independent approaches are essential in order to gain more information about the *in-situ* (eco)physiology of these microbes.

However, it is challenging to link the identity of microbes with a specific set of metabolic processes in order to shed light on their potential role in the environment. Microautoradiography coupled to fluorescence *in situ* hybridization (MAR-FISH) is a powerful single-cell tool to study particular physiological properties of different microbes. Short incubation times with low concentrations of radiolabeled tracers allow an assessment of the uptake of defined substrates at close to *in-situ* conditions. Since MAR-FISH sample preparation and evaluation is rather laborious, most studies so far have focused on a small numbers of tracers and/or microbial populations (Pérez *et al.,* 2010, Salcher *et al.,* 2010), which does not allow to draw

68 conclusions about substrate partitioning by sympatric bacterioplankton taxa.
69 Moreover, as FISH-based studies depend on the availability of specific rRNA-
70 targeted oligonucleotide probes, many investigations using MAR-FISH have only
71 analyzed uptake of broad phylogenetic lineages rather than of species- to genus-like
72 bacterial populations (Pérez *et al.,* 2010, Salcher *et al.,* 2010). Leucine (Leu) and
73 thymidine (TdR) are widely used as precursors for biomass synthesis and cell growth
74 (Kirchman 2001, Cottrell and Kirchman 2003, Pérez *et al.,* 2010). However, bacterial
75 production cannot be solely assessed by using Leu or TdR, as only a minor proportion
76 of obviously active bacteria is able to take up TdR (Pedros-Alio and Newell 1989,
77 Pérez *et al.,* 2010), probably due to a lack in TdR transport systems or preferred *de*
78 *novo* synthesis. Moreover, substantial differences in the incorporation of TdR and Leu
79 were found for different freshwater microbial populations (Pérez *et al.,* 2010).

80 Substrate based niche partitioning of freshwater bacterioplankton populations
81 has also been proposed for acetate, glucose, and Leu incorporation (Buck *et al.,*
82 2009), as well as for N-acetyl-glucosamine and Leu uptake (Eckert *et al.,* 2012). Such
83 a specialization is in contrast to the idea of non-selective microbial substrate
84 assimilation ('mixed-substrate-growth') under oligotrophic conditions (Egli 2010). By
85 contrast, the 'paradox of the plankton' concept (Hutchinson 1961) suggests niche
86 differentiation of coexisting species in an seemingly unstructured environment such as
87 freshwaters where competitive exclusion could otherwise not explain the high
88 observed microbial diversity.

89 We compared the substrate uptake spectra of prominent bacterioplankton
90 populations in an oligo-mesotrophic prealpine lake (Lake Zurich, Switzerland).
91 Specifically, we tested if these microbial lineages mainly competed for the same

substrates or if their co-occurrence might be also related to specific substrate niches. Fourteen monomeric radiolabelled tracers were used to determine microbial acquisition of various low-molecular-weight (LMW) organic compounds and substrate specialization of different co-occurring microbial populations. As *Betaproteobacteria* were found to incorporate a wide range of the applied LMW tracers, we designed new oligonucleotide probes targeting the 16S rRNA of species-like betaproteobacterial populations to study the most active microbes in more detail.

Materials and methods

Study site and sampling

Lake Zurich is a large, deep (136 m), prealpine, oligo-mesotrophic lake, characterized by persistent annual blooms of the toxic cyanobacterium *Planktothrix rubescens* (Posch *et al.,* 2012). Sampling took place on 16 September 2009 at the deepest part of the lake (136 m). Vertical profiles of temperature, conductivity, turbidity, oxygen, and chlorophyll *a* content were recorded using a YSI multiprobe (Yellow Springs Instruments, model 6600) and a bbe fluoroprobe (TS-16-12, bbe Moldaenke GmbH), respectively. The fluoroprobe was calibrated to distinguish different phytoplankton groups (i.e., diatoms, cryptophytes, and green algae) and *P. rubescens* according to their repective pigment fluorescence spectra (Beutler *et al.,* 2002, Salcher *et al.,* 2011a). Water samples were taken from 0, 5, 10, 20, 30, 40, 60, 80, and 100 m depth for flow cytometry and FISH analyses. Forty ml of water were fixed with formaldehyde (2% final concentration) for flow cytometry, and 5-10 ml were fixed with freshly prepared buffered paraformaldehyde (pH 7.4, 2% final concentration) for CARD-FISH analyses. Chemical parameters (dissolved organic carbon, dissolved phosphorus, nitrate, and ammonium concentrations) were determined by the Zurich Water Supply using standard techniques.

Sequence analysis of 16S rRNA genes, and probe design

Two hundred fifty ml of unfixed water samples from 10 m, 12.5 m, and 15 m depth from 1 October 2007 were used for the construction of 3 16S rRNA gene clone libraries (see Salcher et al. 2011b, and Van den Wyngaert et al. 2011 for more details). Phylogenetic analyses were performed with the ARB software package (Ludwig *et al.,* 2004) using the SILVA database SSU Ref release 106 (Pruesse *et al.,*

2007). Sequences affiliated with different clades of *Betaproteobacteria* (>1200 nucleotides and with good quality scores) were selected for bootstrapped maximum likelihood analyses (RAxML, 100 repetitions) on a web server (Stamatakis *et al.,* 2005). Nodes with bootstrap values <50% were collapsed into multifurcations. Oligonucleotide probes were designed for four species- to genus-like betaproteobacterial populations with the respective ARB tools. The probes were checked *in silico* against the database of the ribosomal database project (http://rdp.cme.msu.edu), and tested on environmental samples at different formamide concentrations until highest specificity was achieved (Table 1). A set of several helper and competitor oligonucleotides was designed to enhance the accessibility and specificity of probes bet VI-994 and GKS98-584, respectively (Table 1, Amann and Fuchs 2008).

Abundances of auto- and heterotrophic prokaryotes

Abundances of bacteria and autotrophic picocyanobacteria were determined by an inFlux V-GS cell sorter (Becton Dickinson) equipped with a UV (355 nm) and blue (488 nm) laser. All samples were stained with 4',6-Diamidino-2-phenylindole (DAPI, 1 μg mL^{-1} final concentration), and scatter plots of DAPI fluorescence vs. 90° light scatter were analyzed with the software FlowJo 7.2.2. (Tree Star).

Abundances of different bacteria

CARD(Catalyzed reporter deposition)-FISH with fluorescein-labeled tyramides was carried out as previously described (Sekar *et al.,* 2003) with slight modifications for probe LD12-121 (Salcher *et al.,* 2011b). Horseradish peroxidase (HRP) labeled oligonucleotide probes used for community analysis were: EUB I-III (all *Bacteria*), HGC69a (*Actinobacteria*), BET42a (*Betaproteobacteria*), ALF968

147 (*Alphaproteobacteria*), CF319a (mostly *Flavobacteriales,* phylum *Bacteroidetes*),
148 GAM42a (*Gammaproteobacteria*) (for details see Amann and Fuchs 2008), Ver47
149 (*Verrucomicrobia (Arnds et al., 2010)*), ac1-853 (acI cluster of *Actinobacteria*;
150 (Warnecke *et al.,* 2005)), LD12-121 (uncultured LD12 *Alphaproteobacteria*; (Salcher
151 *et al.,* 2011b)), Cyc715 (*Cyclobacteriaceae*, *Sphingobacteriales, Bacteroidetes*;
152 (Eckert *et al.,* 2012)), LD2-739 (LD2 cluster of *Saprospiraceae*, *Sphingobacteriales*,
153 *Bacteroidetes*; (Pernthaler *et al.,* 2004)), R-BT065 (*Limnohabitans* sp.,
154 *Betaproteobacteria*; (Šimek *et al.,* 2001)), PnecB-23S-166 (*Polynucleobacter*
155 *acidiphobus* and *P. difficiles*, *Betaproteobacteria*), PnecC-445 (*P. necessarius* spp.
156 *asymbioticus*, *Betaproteobacteria*; (Hahn *et al.,* 2005)), LD28-1017 (uncultured LD28
157 tribe, *Betaproteobacteria*, (Salcher *et al.,* 2011a)), and the newly designed probes
158 listed in Table 1. CARD-FISH stained filters were analyzed by fully automated high-
159 throughput microscopy (Zeder and Pernthaler 2009). All images were analyzed with
160 the freeware image analysis software ACMEtool (technobiology.ch), and interfering
161 autofluorescent cyanobacteria or debris were individually subtracted from hybridized
162 cells. At least 10 high quality images or >1000 DAPI stained bacteria were analyzed
163 per sample.

164 *Tracer incubations and MAR-FISH*

165 MAR assays and bulk uptake measurements with 14 radiolabelled tracers
166 (Table 2, all purchased from Amersham, GE Healthcare and American Radiolabeled
167 Chemicals) were performed on water samples from 5 m depth. The amino acid
168 mixture (AA) is composed of 15 individual AAs (i.e., Ala, Arg, Asp, Glu, Gly, His,
169 Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, and Val). Each substrate was added to a
170 subsample of 6 ml at a final concentration of 10 nmol l^{-1}, and subsamples were

171 incubated for 2 h at *in situ* temperature in the dark in triplicates plus 2 prefixed
172 controls. Bulk uptake rates were determined from 5 ml of these samples with a
173 scintillation counter (Tri-Carb 3170TR/SL, PerkinElmer) according to the cold TCA
174 method described in Kirchman (2001). The remaining 1 ml of subsample was filtered
175 onto white polycarbonate filters (Millipore), processed as described in Salcher et al.
176 (2011b) and manually evaluated with a Zeiss Microscope at bright field illumination
177 and blue excitation. At least 100 hybridized cells were inspected per sample.
178 Filamentous bacteria, picocyanobacteria, and *Planktothrix rubescens* filaments were
179 determined from several preparations and counts for a single tracer were pooled.

180 *Statistical analysis*

181 Agglomerative hierarchical clustering was done to identify significant clusters
182 of either tracers or populations based on Pearson's correlation coefficients using the
183 unweighted pair-group average. Data were normalized by log(x+1) transformation. A
184 mantel test (1000 permutations) was used to test for a significant relationship between
185 depth distribution and tracer uptake of different bacterial populations. Only species- to
186 family-like bacterial populations and all tracers except AA were included in cluster
187 computation. Clustering was done with XLSTAT ADA (Addinsoft).

Results

Lake Zurich was thermally stratified with a metalimnetic oxygen minimum (<4 mg O_2 l^{-1}) between 16-18 m depth in 16 September 2009 (Fig. 1). Dissolved organic carbon (DOC) was present in very low concentrations (1.3-1.7 mg l^{-1}) with a slight tendency to decrease with depth, while dissolved phosphorus (PO_4-P) was below the limit of detection (2 µg l^{-1}) until 40 m depth and increased with depth. Nitrate (NO_3-N) was depleted in the epilimnion and increased in the hypolimnion, whereas ammonia (NH_4-N) showed a pronounced peak (70 µg l^{-1}) in 12 m depth. The dominant primary producer, the filamentous cyanobacterium *Planktothrix rubescens*, had a distinct maximum in 12 m depth, while small picocyanobacteria (presumably *Synechococcus* sp.) were abundant in the water layers above. Diatoms and chlorophytes were present in low numbers as samples were taken at the decline of a diatom summer bloom usually observed in August (data not shown). The highest abundances of heterotrophic bacteria were found in the warm, light penetrated epilimnion (0-10 m depths) with a slight decline in 5 m depth. The majority of microbes belonged to the low nucleic acid (LNA) fraction (77% of all bacteria, data not shown), whereas high nucleic acid (HNA) cells became more abundant in the deep hypolimnion (i.e., 30% in 40-100 m vs. 17 % in the epilimnion). Thus, the epilimnion was dominated by small sized cells.

Very small bacteria (i.e., ultramicrobacteria with a cell length of ≤1 µm) affiliated with the ac1 clade and other lineages of *Actinobacteria* were more frequent in the epi- than in the hypolimnion (Fig. 1). Moreover, LD12 ultramicrobacteria (*Alphaproteobacteria*) were only present in high numbers in 0-10 m depths (up to 0.4 x 10^6 cells ml^{-1} in the surface), and 10 times less abundant between 20-100 m depth.

Other *Alphaproteobacteria*, as well as *Gammaproteobacteria* and *Verrucomicrobia* contributed <1% to the total assemblage and were therefore excluded from further analyses. *Betaproteobacteria* and *Bacteroidetes* (as hybridized with probes CF319a and Cyc715) could be detected throughout the whole water column (between 4-17% of *Bacteria*).

Incorporation profiles of different bacterial populations.

The different radiolabelled tracers were incorporated by 1 to 44% of all DAPI-stained microbes, respectively (Fig. 2A). While Fru, Lys, Arg, Asp, Ser, Gly, and Ala (abbreviations see table 2) were incorporated by <4% of all cells, Ace, TdR, Glc, Gln, and Glu were more frequently taken up (8-26%). Highest incorporation was detected for Leu (40%) and AA (44%). Picocyanobacteria did not take up any of the offered substrates (Fig. 2B), while *P. rubescens* was highly efficient in the incorporation of Asp, Gln, Glu, Ser, Gly, and Ala (64-98% of filaments, Fig. 2C). Ace and Glc were also incorporated by one third and one quarter of all *P. rubescens* filaments, respectively (Fig. 2C).

Actinobacteria of the ac1 tribe mainly incorporated TdR, Glc, AA, and Leu (Fig. 2D), as opposed to microbes affiliated with the LD12 cluster (*Alphaproteobacteria*), which showed only a weak incorporation of these tracers (except AA, Fig. 2E). These microbes were on the other hand highly active in Gln and Glu uptake. *Betaproteobacteria* in general incorporated most of the offered tracers to a high extent; only for Fru, Ser, Gly, and Ala no incorporation could be detected (Fig. 2F). *Bacteroidetes* showed a contrasting pattern: while microbes targeted by probe CF319a (mainly *Flavobacteriales*) were seemingly inactive (Fig. 2G), bacteria affiliated with *Cyclobacteriaceae* (*Sphingobacteriales*) positively selected Arg, Glc,

and Leu (65-85%), and to a lesser extent TdR, Gln and Glu (24-34%, Fig. 2H). A comparable pattern was detected for filamentous DAPI stained microbes (Fig. 2I): Almost all filaments (99%) took up Arg and 51-80% were positive for Leu, TdR, Glc and Fru. A large proportion (44.4%) of these filaments was affiliated with the ubiquitous LD2 cluster of *Saprospiraceae (Sphingobacteriales, Bacteroidetes,* data not shown).

Ratios of active cells in populations to all active Bacteria*.*

A ratio of population specific activity versus activity of all bacteria (as hybridized by probe EUB) was calculated by summing up the abundances of MAR+ cells of all 14 tracers (Fig. 3). The most abundant microbes (i.e. *Actinobacteria* and therein the ac1 cluster and LD12 bacteria) mirrored the activity of the total assemblage, while the active cell fraction within *Betaproteobacteria* was 2.5 times above the community average. Microbes hybridized by probe CF319a were 8 times less active, while *Cyclobacteriaceae* were slightly above the community average.

Betaproteobacterial populations.

By applying 8 species- to genus-specific probes (i.e., targeting genotypes with >98% and >95% 16S rRNA gene sequence similarity) we succeeded to identify the vast majority (84 ± 11 %) of all *Betaproteobacteria* along the depth profile (Fig. 4). In the sampling depth for the MAR-FISH analysis (5 m), 94 % of all *Betaproteobacteria* were covered by the specific probes. The most abundant *Betaproteobacteria* were affiliated with *Limnohabitans* sp. (R-BT), followed by the species-like tribe LD28. *Polynucleobacter acidiphobus* and *P. difficiles* (PnecB) mainly populated the epilimnion, while *Polynucleobacter necessarius* ssp. *asymbioticus* (PnecC) were only

present in low densities. The 4 newly designed probes target small clusters within bet I, bet VI, and the whole tribe bet III (GKS98). The species-like population bet I-B, closely related to *Rhodoferax* sp. and *Albidiferax* sp., was present throughout the water column, while microbes of the species-like cluster bet I-C, relatives of *Curvibacter* sp., were restricted to the epilimnion. A similar spatial pattern was found for a species-like cluster within the bet VI lineage. Microbes affiliated with the genus-like (i.e., >96.6% 16S rDNA sequence similarity) bet III cluster, related to *Parapusillimonas granuli*, were present in all depths, albeit in very low abundances. These microbes, as well as PnecC were not further analyzed by MAR-FISH due to their very low numbers.

Five of the six analyzed betaproteobacterial subpopulations shared several preferred substrates, i.e., TdR, Ace, AA, Gln and / or Glu, and Leu (Fig. 5). On the other hand, Glc, Lys, Arg, Asp, Ser, and Ala were variable for each population. Fru and Gly were not or only slightly taken up by any of the populations.

Nine of the 14 substrates were incorporated by more than 50% of all R-BT (54-95%), and four more by at least one sixth of the population. Only Fru was not incorporated by R-BT (Fig. 5a). Microbes affiliated with bet I-B incorporated all substrates to a certain extent (Fig. 5b). High uptake (>80%) was observed for 7 tracers, medium uptake (22-44%) for 6 more tracers, and low uptake for Fru (15%). Very high uptake of 5 radiolabeled tracers was observed for the bet I-C population, while 5 other substrates were not incorporated at all (Fig. 5c). Microbes of the LD28 tribe did not take up any of the offered tracers (Fig. 5d), while PnecB and bacteria affiliated with bet VI incorporated 10-12 substrates (Fig. 5e & f). The latter two

282 populations showed a comparable pattern with high uptake of Ace, AA, Arg, Gln,
283 Glu, Leu, TdR, and Glc.

284 *Clustering of populations and tracers.*

285 Agglomerative hierarchical clustering based on Pearson's correlation
286 coefficients identified 4 significant clusters of bacterial populations according to their
287 depth distribution (Fig. 6a). Populations with a clear surface preference (LD12, bet
288 VI, and bet I-C) were separated from others with a more homogeneous distribution
289 over depth. Clustering based on similarities of microbial uptake spectra (Fig. 6b)
290 resulted also in 4 significant clusters, albeit with a significantly different grouping
291 (i.e., the absence of a significant correlation between the data sets in a Mantel test,
292 p=0.131). *P. rubescens* was clearly separated from all heterotrophic microbes in its
293 substrate uptake pattern, and incorporation patterns of LD28 *Betaproteobacteria* were
294 different from all other investigated bacterial groups. *Alphaproteobacteria* of the
295 LD12 clade clustered weakly with the betaproteobacterial tribes R-BT, bet I-B,
296 PnecB, and bet VI. Microbes affiliated with bet I-C differed from all other bacteria,
297 and a cluster of ac1 *Actinobacteria* and *Cyclobacteriaceae* could be distinguished.
298 Specialized bacteria with an incorporation of only some tracers were affiliated with
299 LD12, ac1, *Cyclobacteriaceae*, and bet I-C. On the other hand, all other populations
300 affiliated with *Betaproteobacteria* (except for LD28) and *P. rubescens* incorporated a
301 wide range of substrates, pointing to a more generalist lifestyle. Three significant
302 clusters of substrates that tended to be consumed together could be identified (Fig.
303 6c). The first deeply branching cluster represented tracers which were incorporated to
304 a high extent exclusively by *P. rubescens* (i.e., Asp, Ala, Ser, and Gly). The second
305 cluster included the remaining tracers. Therein, Glc was clearly separated from all

306 other substrates. Glu was incorporated by almost all analyzed microbial populations,
307 followed by Leu, Glc, Gln, and TdR. On the other hand, Lys, Asp, Ala, Ser, and Gly
308 appeared to be more specialized substrates, and Fru was never incorporated by more
309 than 25% of any of the analyzed populations.

Discussion

Phytoplankton as major sources of low molecular weight substances.

Dissolved organic matter (DOM) in freshwaters is composed of a multitude of different substances. The fraction of LMW DOM mainly consists of amino acids (AAs), carbohydrates (CHs), and carboxylic acids (CAs), molecules that can be rapidly utilized by microbes with turnover rates of several hours (Sundh 1992, Rosenstock and Simon 1993). Dissolved free AAs form a substantial pool (i.e. 12-32 % in Lake Constance) of the labile DOM in freshwaters with strong seasonal variations (Weiss and Simon 1999). Ace is often the most important CA in lakes, while Glc and Fru were found to be equally abundant CHs (Bertilsson and Tranvik 1998, Zotina *et al.,* 2003, Berggren *et al.,* 2010). Previous studies on Lake Zurich aimed to quantify the concentrations of AAs (i.e., ~10-25 nM of individual AAs, ~100 nM in total), CHs (~20 nM), and CAs (~1 μM) (Meon and Jüttner 1999, Zotina *et al.,* 2003). We added all tracers in final concentrations of 10 nM which corresponds approximately to the *in-situ* conditions, except for acetate, which was added in tracer amounts in our assays.

Phytoplankton blooms are a main source of labile DOM readily available for microbes, as a substantial fraction of extracellular phytoplankton products (EPP) such as AAs, CHs, or CAs is released by algae (Hama and Handa 1987). Although the majority of EPP are polymers (Giroldo and Vieira 2005), bacteria were found to utilize algal LMW exudates more rapidly and to a much higher extent (Giroldo *et al.,* 2007). The exudation of e.g., CHs is both, species- and growth phase dependent, with highest amounts usually released during late stationary phase (Giroldo and Vieira 2005). Our experiments were conducted at the late phase of a phytoplankton summer

bloom dominated by chlorophytes, chrysophytes, and diatoms; therefore, substantial EPP release can be expected. The observed high number of active cells within different microbial populations (Figs. 2, 5) might thus be also a result of microbes already being pre-adapted to increased availability of LMW substrates. Glc, besides mannose / xylose, released by a freshwater diatom was found to be the principle source of CHs supporting bacterial growth (Giroldo *et al.,* 2007). In our study, Glc was mainly incorporated by ac1 *Actinobacteria*, *Cyclobacteriaceae*, R-BT, and filamentous bacteria (Figs., 2, 5), microbes that are typically associated with algal blooms (Šimek *et al.,* 2008, Salcher *et al.,* 2010, Eckert *et al.,* 2012).

Substrate niche differentiation of the dominant microbes.

The most abundant microbes in Lake Zurich (i.e. ac1 *Actinobacteria* and LD12 *Alphaproteobacteria*, Fig. 1) showed strikingly different substrate preferences (Figs. 2d, 2e). Therefore, these apparently highly specialized ultramicrobacterial populations might co-exist in the water column by largely avoiding competition for LMW DOM compounds. The uptake pattern of ac1 *Actinobacteria* suggested adaptation to phytoplankton exudates (Glc and Leu) in combination with rapid cell division (TdR). A nearly complete genome was recently obtained from a single sorted cell of the ubiquitous ac1-B1 tribe (Garcia *et al.,* 2012). This streamlined genome included several genes encoding for AA transporters (e.g., ABC transporters for branched-chain AAs such as Leu), but lacked a transporter for Glc. This is in contrast to our results (Fig. 2d), as well as to previous findings of Glc uptake by ac1-B1 (Buck *et al.,* 2009). Other successful planktonic microorganisms with reduced genomes such as the marine SAR11 clade or *Polynucleobacter* sp. are highly diversified, with physiologically distinct ecotypes adapted to specific environmental conditions

358 (Schwalbach *et al.,* 2010, Hahn *et al.,* 2012b). Such ecotype diversification might also
359 be the case for ac1 *Actinobacteria*.

360 In contrast, LD12 *Alphaproteobacteria* only weakly incorporated Leu, TdR,
361 and Glc, but were instead specialized on Gln and Glu (Fig. 2e, Salcher *et al.,* 2011b).
362 These AAs are rich in nitrogen and their α-amino groups can be used for the
363 biosynthesis of other AAs (Lengeler *et al.,* 1999). Therefore, LD12 microbes seemed
364 to be well adapted to nitrogen depletion in Lake Zurich during summer (Salcher *et al.,*
365 2011b). LD12 *Alphaproteobacteria* can be regarded as typical oligocarbophils
366 (Salcher *et al.,* 2011b), comparable to their marine sister group SAR11 (Giovannoni
367 *et al.,* 2005). Since these bacteria were almost 3 times more abundant 2 weeks earlier
368 (Salcher *et al.,* 2011b), the population likely was already in decline at the sampling
369 date. As their level of AA incorporation was found to be tightly linked to their
370 seasonal population development (Salcher *et al.,* 2011b), they might not have been at
371 the peak of their activity. A recent study conducted in brackish waters of the Gulf of
372 Gdańsk (Baltic Sea) revealed that up to one quarter of LD12 bacteria actively
373 incorporated TdR, particularly at times of freshwater influx (Piwosz *et al.,* accepted).

374 *Differences within* Bacteroidetes.

375 Three lineages affiliated with *Bacteroidetes* were analyzed in our assay, i.e.
376 *Flavobacteriales* targeted by probe CF319a, *Cyclobacteriaceae* (*Sphingobacteriales*)
377 (Eckert *et al.,* 2012), and filamentous microbes most of which were affiliated with the
378 ubiquitous LD2 tribe of *Saprospiraceae* (*Sphingobacteriales*) (Schauer *et al.,* 2006).
379 Microbes targeted by probe CF319a did not incorporate any of the offered substrates
380 (Fig. 2g). This does not imply that these microbes were metabolically inactive.
381 Marine and brackish *Flavobacteriales* preferably incorporated high molecular weight

382 DOM such as proteins or chitin (Cottrell and Kirchman 2000). Freshwater
383 *Flavobacteriales* were repeatedly reported to be only marginally involved in AA
384 turnover (Pérez and Sommaruga 2006, Salcher *et al.,* 2010), but could be
385 experimentally enriched with chitin (Beier and Bertilsson 2011) and incorporated the
386 mono- and dimeric chitin units N-acetyl-glucosamine and di-N-acetyl-glucosamine
387 (Beier and Bertilsson 2011, Eckert *et al.,* 2012). The freshwater bacterium
388 *Flavobacterium johnsoniae* strain A3 preferentially utilized oligopeptides and
389 proteins over individual AAs and had a higher specific affinity for di- and
390 polysaccharides than for Fru (Sack *et al.,* 2011). Our results support the hypothesis of
391 *Flavobacteriales* being specialized on substrates other than LMW DOM (Fig. 2g).
392 *Cyclobacteriaceae* (*Sphingobacteriales*), on the other hand, were specialized
393 on Glc, Arg, and Leu incorporation (Fig. 2h). The applied FISH probe targets almost
394 the complete family and was designed to quantify *Cyclobacteriaceae* that were not
395 detected by the general probe CF319a during a phytoplankton spring bloom in Lake
396 Zurich (Eckert *et al.,* 2012). These microbes were most abundant during the late
397 bloom phase and were highly involved in turnover of N-acetyl-glucosamine (Eckert *et*
398 *al.,* 2012). Cultivated members of this lineage (*Algoriphagus* sp.) were able to grow
399 on Glc as sole carbon source (Liu *et al.,* 2009), pointing to a dependency on
400 phytoplankton exudates.
401 Filamentous microbes, predominantly affiliated with the LD2 tribe of
402 *Saprospiraceae* (Schauer *et al.,* 2006) specialized on the uptake of Arg, Leu, TdR,
403 Glc, and Fru (Fig. 2i). Moreover, high TdR incorporation indicated rapid cell division.
404 Filaments often reach maximal densities at late phases of algal blooms when protistan
405 grazing pressure is high (Pernthaler *et al.,* 2004, Eckert *et al.,* 2012), as this
406 morphology protects from flagellate predation (Pernthaler 2005, Schauer *et al.,* 2006).

However, the intense uptake of AAs and sugar monomers suggested that filaments might also have directly profited from algal exudates.

Active betaproteobacterial populations.

Using a set of 8 species- to genus like probes we were able to identify almost all *Betaproteobacteria* (Fig. 4), which were the most active microbial group with respect to LMW assimilation (Fig. 3). Cryptophytes and chlorophytes, both of which were present at the sampling date, are known to release EPP that support the growth of *Betaproteobacteria* (R-BT, PnecB, and others), especially during late bloom phases (Šimek *et al.,* 2011). Closely related populations (i.e., R-BT, bet I-B, and bet I-C, all belonging to the bet I lineage, Fig. 4) differed drastically in their incorporation patterns (Fig. 5), and thus seemed to largely avoid competition for the same substrates. On the other hand, microbes affiliated with PnecB and bet VI showed similar uptake whereas they are not phylogenetically closely related (Figs. 4, 5). In general, R-BT, PnecB, and microbes of the uncultured bet I-B and bet VI tribes had very broad tracer uptake spectra (Figs. 5, 6), pointing to a more generalist life style and the preparedness to grow on a wide range of substrates. An 'opportunistic' life strategy has been previously postulated for R-BT, based on their fast growth that is tightly linked to phosphorus and algal-derived carbon sources (Šimek *et al.,* 2005, Šimek *et al.,* 2008, Šimek *et al.,* 2011), and their sensitivity to grazing by protists (Jezbera *et al.,* 2006). Bet I-C on the other hand seemed to be more specialized, e.g., Leu and Glu were the only preferred AAs. Bacteria of the uncultured LD28 (bet IV) tribe of *Betaproteobacteria* are close relatives of the marine OM43 lineage and other methylotrophs, e.g., *Methylotenera* sp. (Fig. 4). Marine OM43 are commonly associated with diatom blooms (Sekar *et al.,* 2004, Morris *et al.,* 2006) and utilize C1

substrates such as methanol, formate, and formaldehyde (Giovannoni *et al.,* 2008). High numbers of LD28 were detected in deep water beneath the chlorophyll *a* maxima in Lake Zurich (Salcher *et al.,* 2011a) and a small prealpine lake (Salcher *et al.,* 2008). In the present study, LD28 did not incorporate any of the offered substrates (Fig. 5d), probably due to a methylotrophic life style.

Photoheterotrophy of cyanobacteria – powerful competitors for heterotrophs.

Picocyanobacteria (presumably *Synechococcus* sp.) did not incorporate any of the offered substrates (Fig. 2b) and therefore likely were predominantely phototrophic. This differs from observations in marine waters where they could assimilate AAs and ATP (Zubkov et al., 2003, Michelou et al., 2011). By contrast, the filamentous cyanobacterium *Planktothrix rubescens*, the dominant primary producer in Lake Zurich (Posch *et al.,* 2012), took up a variety of AAs and even incorporated Glc and Ace (Fig. 2c), as also observed previously (Zotina et al., 2003, Walsby and Jüttner 2006, Horňák et al., 2012). In particular, nitrogenous organic compounds may fuel most of the nitrogen demand of this non N_2-fixing cyanobacterium (Zotina et al., 2003). While most of the preferentially incorporated AAs (esp. Ala, Gly, Ser, and Asp) were not or only marginally taken up by heterotrophic bacteria (Figs. 2, 5, 6), Gln and Glu were highly incorporated by both microbial guilds. Therefore, *P. rubescens* can be a potent competitor of heterotrophic microbes for DOM. This competition might be even more significant when considering biomass specific substrate uptake, as *P. rubescens* represents 10 times more biomass than bacteria in the epilimnion of Lake Zurich (Van den Wyngaert *et al.,* 2011). Moreover, unlike other phototrophs, this cyanobacterial species has only very low DOM excretion rates (Feuillade *et al.,* 1988), which makes it doubtful that it may also represent a source of dissolved substrates for heterotrophic bacteria in the photic zone of the lake.

Substrate specialization: a common feature of freshwater microbes?

We identified two general strategies of LMW DOM acquisition by planktonic bacteria: For one, there was uptake of a broad range of substrates by several generalist and ecophysiological versatile betaproteobacterial populations (Figs. 5, 6). Such preparedness for growth on mixed substrates has been postulated as a general rule for microbes in oligotrophic environments (Egli 2010). However, our results also indicated a high degree of specialization, as the most abundant microbes (i.e., ac1 *Actinobacteria* and LD12 *Alphaproteobacteria*), both of which are known for their oligotrophic life style (Salcher *et al.,* 2011b, Garcia *et al.,* 2012), showed no overlap in their LMW substrate acquisition spectra (Fig. 2). Moreover, substrate selectivity was also found for *Bacteroidetes* (CF, *Cyclobacteriaceae*, and filaments) as well as for two populations of *Betaproteobacteria*. This suggests that any generalized estimate of the *in situ* activity of freshwater microbes (or their active fraction) by single radiolabeled tracers such as Leu or TdR (e.g., Cottrell and Kirchman 2003, Pérez *et al.,* 2010) may be biased. For example, at least one of the tested substrates was taken up by >90% of cells from the various betaproteobacterial populations (except for LD28, Fig. 5), but the preferred substrates differed (i.e., Glc for R-BT, Gln for PnecB, bet I-B, and bet VI, Leu for bet I-C). Therefore, all these populations must be regarded as highly active.

The observed physiological niche separation might also be an important reason for the coexistence and considerable diversity of planktonic bacterial taxa in freshwater lakes. Interestingly, microbial populations with similar substrate uptake patterns also had different vertical distributions in the lake (Fig. 6a,b), hinting at other niche separation factors such as oxygen content or temperature. Our results nevertheless provide an example for coexistence mediated by substrate based

ecological diversification, as was recently suggested for the degradation of complex polysaccharides (Teeling *et al.,* 2012). Such an ecophysiological niche separation might moreover be regarded as a (partial) solution for the classical 'paradox of the plankton' (Hutchinson 1961) with respect to heterotrophic pelagic bacteria.

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771 Titles and legends to figures

772 Figure 1: Vertical profiles of physical, chemical, and biological parameters in Lake
773 Zurich at the sampling date. DOC: dissolved organic carbon; PO_4-P: phosphate-P,
774 NH_4-N: ammonium-N, NO_3-N: nitrate-N; ac1: ac1 tribe of *Actinobacteria*; ACT:
775 *Actinobacteria*; LD12: LD12 tribe of *Alphaproteobacteria*; BET:
776 *Betaproteobacteria*; CF: *Flavobacteriales*; CYC: *Cyclobacteriaceae*; EUB:
777 *Bacteria*.

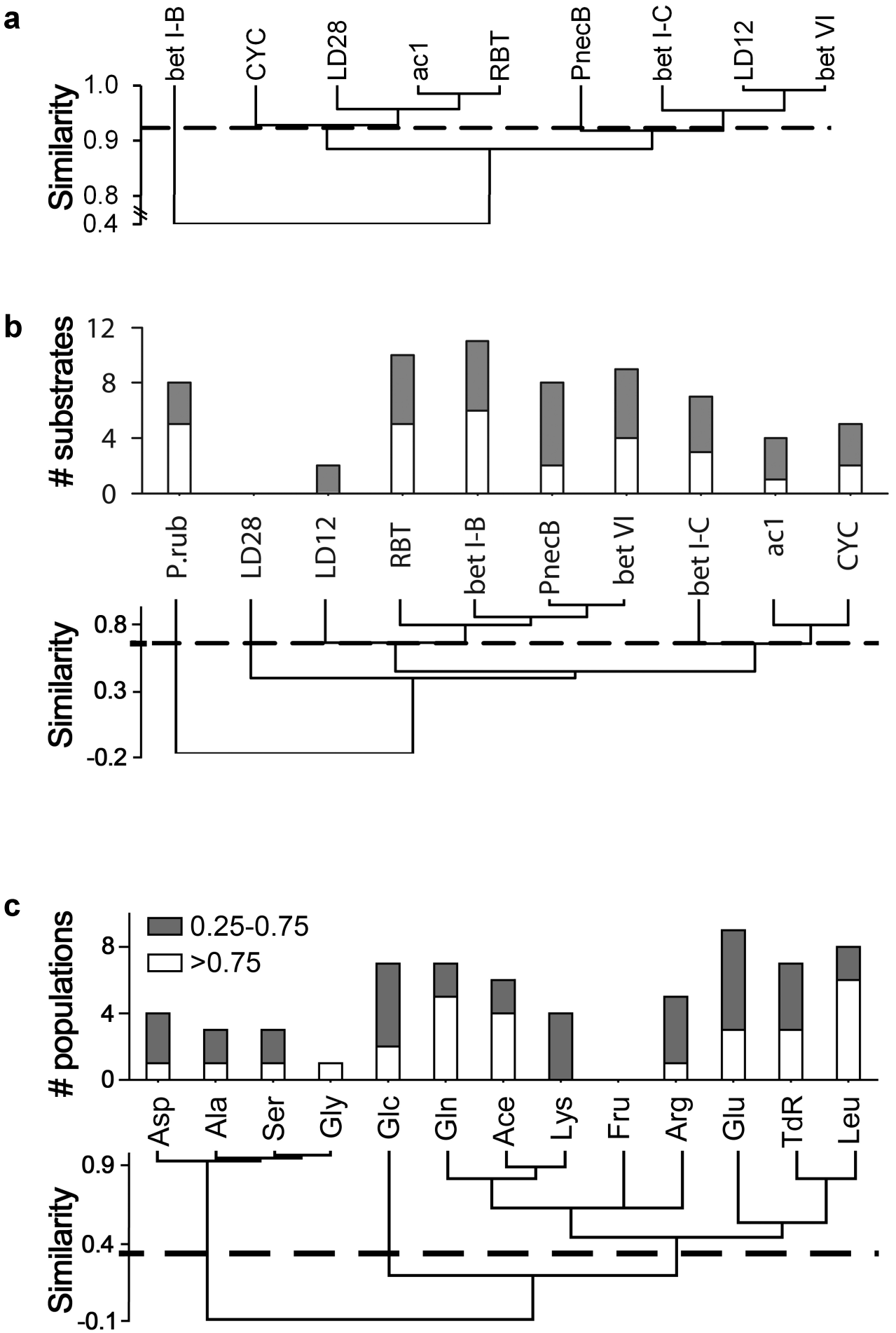
778 Figure 2: Ecophysiological MAR-FISH assay with 14 radiolabeled substrates. Means
779 and standard errors (n = 3) of the percentage of all DAPI stained bacteria (a),
780 picocyanobaceria (b), the cyanobacterium *Planktothrix rubescens* (c), ac1
781 *Actinobacteria* (d), LD12 *Alphaproteobacteria* (e), *Betaproteobacteria* (f),
782 *Flavobacteriales* targeted by probe CF319a (g), *Cyclobacteriaceae* (h), and
783 filamentous bacteria (i) with active tracer incorporation are shown. See Table 2
784 for abbreviations of tracers. Scaling in 2a applies to all populations.

785 Figure 3: Ratio of active populations to active *Bacteria* (as hybridized with probe
786 EUB I-III). All tracers were pooled for calculation. Abbreviations see Fig.1.

787 Figure 4: Maximum likelihood tree of the 16S rDNA of Betaproteobacteria and their
788 ubiquitous freshwater lineages (a). Clusters in grey mark populations subjected to
789 CARD-FISH with newly designed or already published probes. b: Vertical
790 distribution of betaproteobacterial populations.

791 Figure 5: Ecophysiological MAR-FISH assay with 14 radiolabeled substrates. Means
792 and standard errors (n = 3) of the percentage of different betaproteobacterial
793 populations with active tracer incorporation are shown. See Table 2 for
794 abbreviations of tracers and Figure 4 for phylogeny and abundance of
795 populations. Scaling in 5d applies to all populations.

Figure 6: (a) Agglomerative hierarchical clustering of bacterial populations based on the depth distribution of their abundances in Lake Zurich. (b) Upper panel: Number of substrates incorporated by a specific population to a very high (>0.75, white) or medium (0.25-0.75, grey) extent. Lower panel: Agglomerative hierarchical clustering of bacterial populations based on substrate incorporation. (c) Upper panel: Number of populations incorporating a specific tracer to a very high (>0.75, white) or medium (0.25-0.75, grey) extent. Lower panel: Agglomerative hierarchical clustering of tracers. Agglomerative hierarchical clustering was based on Pearson's correlation coefficients using unweighted pair-group average. Only species- to family-like bacterial populations and all tracers except AA were computed.


a
bet I-B
CYC
LD28
ac1
RBT
PnecB
bet I-C
LD12
bet VI
Similarity
1.0
0.9
0.8
0.4
b
substrates
12
8
4
0
P.rub
LD28
LD12
RBT
bet I-B
PnecB
bet VI
bet I-C
ac1
CYC
Similarity
0.8
0.3
-0.2
c
populations
0.25-0.75
>0.75
8
4
0
Asp
Ala
Ser
Gly
Glc
Gln
Ace
Lys
Fru
Arg
Glu
TdR
Leu
Similarity
0.9
0.4
-0.1